EUROPEAN COMMISSION
DIRECTORATE-GENERAL
JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives Authorisation

D08-FSQ(2005)D/31316

CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2005-080
Name of Additive:	Phyzyme™ XP 5000L / 5000G
Active Substance:	6-Phytase (EC 3.1.3.26)
Rapporteur Laboratory:	Sächsische Landesanstalt für Landwirtschaft, Leipzig, Germany
Report prepared by:	Jens Schönherr
Report checked by:	Anne-Mette Jensen (CRL-FAA)
Date:	15 December 2005
Report approved by:	Christoph von Holst (CRL-FAA)
Date:	22 December 2005

EXECUTIVE SUMMARY

In the current application authorisation is sought for Phyzyme™XP under the category zootechnical additives, groups 4.a and 4.c, according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use Phyzyme™XP as a digestibility enhancer for chickens, turkeys, laying hens, ducks, piglets, pigs and sows and as a substance that favourably affects the environment by reducing the release of phosphorus into the environment from manure when raising these animals. The feed additive is available in liquid or dry form. The active substance of this feed additive is the enzyme 6-phytase. Its content is expressed in terms of the enzyme activity FTU, where 1 FTU is the amount of enzyme which liberates one µmol of inorganic phosphate from sodium phytate at pH 5.5 and 37°C in one minute. The feed additive which has a target activity of 5000 FTU/g is intended to be mixed into compound feedingstuffs to a final phytase activity of 150–1000 FTU/kg.

For the determination of the phytase activity in the *feed additive* a photometric method is proposed. The method is based on the principle that phytase releases inorganic phosphate from a substrate, which in the presence of a molybdate/vanadate reagent forms a yellow complex. The yellow complex is measured with a spectrometer and the inorganic phosphate is quantified with a phosphate standard curve.

For the determination of the phytase activity in *premixtures* the applicant proposes a photometric method, which is based on the same principle as mentioned above. The protocol contains an additional clean-up step by ion-exchange, in order to separate endogenous phosphate in the sample. The relative standard deviation for repeatability (RSD_r) was 7.3 %, obtained on the measurement of a test sample with a phytase activity of 358 FTU/g.

For the determination of the phytase activity in *feedingstuffs* two very similar photometric methods are proposed which are based on the same principle as mentioned above. For the determination of phytase activity in feedingstuffs containing the *liquid* form of Phyzyme™XP the method is identical to the method used for premixtures. In contrast, the analytical protocol for the analysis of feed samples containing the *dry* form of Phyzyme™XP requires the determination of endogenous phosphate in the feed samples prior to the enzymatic reaction. The amount of endogenous phosphate is taken into account when measuring the phytase activity in the corresponding feed sample. For both methods the applicant reported an RSD_r of 5.3 %, obtained on samples with an appropriate phytase activity of 668 FTU/kg. Also the reported limits of quantification (LOQ) of about 11 FTU/kg were identical for both methods. Though the applicant's methods follow well known principles for the determination of

phytase activity in various matrices, the suitability of these methods for official control purpose cannot be confirmed, given the limited validation data.

Several other analytical methods for the determination of the phytase activity in feedingstuffs exist and have been validated in interlaboratory studies. These include a method proposed by the Association of German Agricultural Analytical and Research Institutes (VDLUFA, Germany), which resulted in a relative between-laboratory standard deviation for reproducibility (RSD_R) of about 12 % for feedingstuffs and an AOAC method which obtained RSD_R values ranging from 14.0 to 27.6 % for feedingstuffs. However, neither of these methods allow for determination of phytase activity from all commercially available phytase products used in feed.

In order to allow for the measurement of the phytase activity in feedingstuffs, regardless of the specific phytase product used, the European Association of Feed Additive Manufacturers (FEFANA) developed a modified method based on the same principle as the applicant's method. The FEFANA method has been validated in an interlaboratory study which was performed on feedingstuffs containing different phytase products including Phyzyme™XP. The obtained values for the between-laboratory RSD_R ranging from 5 to 14 % are considered acceptable for the intended purpose. Therefore, this method which is currently under evaluation to become a standard of the European Committee for Standardisation (CEN), is recommended for official control purposes.

No further testing or validation is required.

KEYWORDS

Phyzyme™XP, phytase, enzyme activity, *Schizosaccharomyces pombe*, digestibility enhancer

TABLE OF CONTENTS

1. BACKGROUND

Phyzyme™XP is a feed additive containing 6-phytase (EC 3.1.3.26), which is produced by *Schizosaccharomyces pombe (ATCC 5233*), as active substance. The applicant proposes to classify the additive belonging to the category 'zootechnical additives, group 4.a and 4.c'.

The intended use (*cf.* EFSA-Q-2005-080) of the current application is to enhance the digestibility of chickens, turkeys, laying hens, ducks, piglets, pigs and sows and as substance that favourable affects the environment by mixing the feed additive into compound feedingstuffs at various concentrations varying from 150 to 1000 FTU/kg.

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and tasks of the CRL-FAA concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to EFSA for each application. For this particular dossier, the suitability of the control methods and validation studies submitted in connection with Phyzyme™XP, *cf.* EFSA-Q-2005-080 was evaluated.

3. EVALUATION

The numbering system under this point refers to the report of the Scientific Committee on Animal nutrition on the revision of the guidelines for the assessment of additives in animal nutrition, adopted on October 22th, 1999 (Guidelines for the assessment of additives in feedingstuffs Part III: Enzymes and Microorganisms).

Description of the methods used for the determination of the criteria listed (cf. pt. 2.5.1 of the Guideline)

Quantitative analysis of active substance in the feed additive

For the determination of the enzyme activity of the active substance (6-phytase) in the feed additive a photometric method is proposed which is based on the release of inorganic phosphate during the hydrolysis of sodium phytate at pH 5.5 and 37°C by the enzyme phytase.

The sample is extracted with acetate buffer and subjected to the enzymatic reaction under defined conditions and after addition of phytate as substrate. The reaction is stopped by addition of acid molybdate/vanadate reagent which also produces a coloured complex with the inorganic phosphate. The colour of the yellow complex is measured at 415 nm and the amount of phosphate quantified through a phosphate standard curve is utilised to calculate the phytase activity in the feed additive.

The method proposed by the applicant is very similar to the VDLUFA-method 'Determination of the phytase activity in enzyme standard materials and enzyme preparations', which has been validated through an interlaboratory study [1] obtaining a relative between-laboratory standard deviation for reproducibility (RSD_R) of 5 %, which is considered acceptable.

Description of the qualitative and quantitative analytical methods for routine control of the active substance in premixtures and feedingstuffs(cf. pt. 2.5.2 of the Guideline).

For the determination of the phytase activity in premixtures and feedingstuffs two slightly different photometric methods were suggested that are both based on the same principle as the method for the determination of the phytase activity in the feed additive.

When analysing premixtures or feed samples, endogenous phosphate, already present in the sample, must be taken into account, before conducting the enzymatic reaction. For the

determination of the phytase activity in feedingstuffs containing the liquid product Phyzyme™XP 5000L and for the analysis of premixtures, the applicant proposes the use of an ion exchange column in order to remove endogenous phosphate. In contrast, for the analysis of feed samples containing the dry product Phyzyme™XP 5000G the applicant proposes a method, in which endogenous phosphate in the sample is measured in a second analysis (blank value) prior to the enzymatic reaction. The result of this analysis is taken into account when calculating the phytase activity of the sample.

Concerning validation data for the method for the determination of the phytase activity in *premixtures* the relative standard deviation for repeatability (RSD_r) was 7.3 %, obtained from the analysis on samples containing 358 FTU/g. For the method for the determination of the phytase activity in *feedingstuffs* the RSD_r was 5.3 %, obtained on samples with a phytase activity of 668 FTU/kg. The values applied to both methods, regardless of whether the method utilises ion-exchange columns or whether the blank value from endogenous phosphate is measured. Also the reported limits of quantification (LOQ) of about 11 FTU/kg were identical for both methods. Though the applicant's methods follow well known principles for the determination of phytase activity in the various matrices, the suitability of these methods for official control purposes cannot be confirmed given the lacking information on the conditions of the validation experiments and the limited validation data available. This particularly is the case for the method using the ion exchange clean-up, since without data on the recovery rate, the accuracy of the method cannot be evaluated.

Several methods for the determination of the phytase activity in premixtures and feedingstuffs exist and have been validated in interlaboratory studies. The methods concerned follow the same principle as above but differ in specific parts of the corresponding analytical protocols.

The AOAC method 2000.12 [2] is a *relative* method, since it quantifies the phytase activity of the samples against a phytase standard with defined activity and not against a phosphate standard (*absolute* method). Both approaches are acceptable, but the use of a phytase standard for the calibration could lead to different results depending on which specific phytase product is used. The method was validated on six different feed samples with a target phytase activity between 200 and 300 FTU/kg, obtaining RSD_r values ranging from 2.5 to 8.6 % and between-laboratory RSD_R values ranging from 14 to 27.6 %.

The VDLUFA method [3], also a relative method, was validated with two feed samples containing 600 FTU/kg and one mineral premixture containing 15676 FTU/kg. The RSD_r ranged from 6.4-7.0% for the feedingstuffs and was 4.9% for the premixture. The between-laboratory RSD_R ranged from 11.1-12.3% for the feedingstuffs and was 8.4% for the premixture.

The AOAC and the VDLUFA methods are limited by the fact that they did not prove to be applicable to different phytase products, thus requiring slight modifications of the protocol to

make it suitable for the determination of phytase activity from various origins. However, since both method methods calibrate against a phytase standard, small differences in the execution of the method in different laboratories can be compensated for by this standard, thereby reducing the between-laboratory variability of the results [4].

In contrast to the AOAC and VDLUFA methods, the FEFANA-method [5] is an absolute method in that it quantifies the phytase activity against a phosphate standard. Therefore, this method does not require the use of a specific phytase product for calibration. In addition, the validation showed that the method can be applied to the analysis of samples regardless of the specific phytase product present in the feedingstuffs. The method has been validated with five feed samples ranging from 750 to 1500 FTU/kg, and containing all the phytase products (including Phyzyme™XP) that are currently commercially available. The RSD_r varied between 3.1 and 13.0% and the between-laboratory RSD_R varied between 5.2 and 14.2% [6]. These precision data are comparable to the performance characteristics of the AOAC and VDLUFA methods and are considered acceptable for the intended purpose. Due to its applicability to the determination of phytase activity in feedingstuffs covering all phytase products currently commercially available, the FEFANA method is recommended for official control purposes.

CHECKLIST FOR SUBMITTED METHODS

		Y	N	N/A	Comments
1.1	Is/Are the method(s) mentioned in Part I (1.- A. Premixtures) accompanied by information on:	X			
	- Sampling method used		X		
	- Percentage mecovery		X		
	- Specificity		X		
	- Accuracy		X		
	- Precision	X			
	- Limit of detection	X			
	- Limit of quantification	X			
	- Validation procedure used		X		
1.2	Is/Are the method(s) mentioned in Part I (Feedingstuffs) accompanied by information on:	X			
	- Sampling method used		X		
	- Percentage mecovery		X		
	- Specificity		X		
	- Accuracy		X		
	- Precision	X			
	- Limit of detection	X			
	- Limit of quantification	X			
	- Validation procedure used		X		

N/A: Not applicable

4. CONCLUSIONS AND RECOMMENDATIONS

The applicant submitted control methods for the determination of phytase activity in the feed additive and for the determination of the two forms of the product in premixtures and feedstuffs. The methods are based on well known and accepted principles but due to limited information on the validation of these methods, the suitability of the proposed methods for official control purposes can not be confirmed.

Several analytical methods for the determination of the phytase activity in feedingstuffs exist and have been validated in interlaboratory studies, obtaining acceptable values for the relative standard deviation for repeatability and reproducibility.

For official control of the phytase activity in feedingstuffs the FEFANA method is recommended, since it has been validated for all commercially available phytase products.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of Phyzyme™XP have been sent to the Community Reference Laboratory for feed additives authorisation. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Bestimmung der Phytaseaktivität in Enzymstandardmaterialien und Enzym-präparaten (Determination of the phytase activity in enzyme standard materials and enzyme preparations)* Method book III of VDLUFA „The chemical analysis of feedingstuffs"; Method Number 27.1.1 ; 4-th Auxiliary supply 1997 ; VDLUFA ISBN 3-922712-66-7, in German

[2] Engelen et al. (2001) J. AOAC Int., 84, 629-633

[3] *Bestimmung der Phytaseaktivität in Futtermitteln und Vormischungen (Determination of the phytase activity in feedstuffs and premixes)* Method book III of VDLUFA „The chemical analysis of feedingstuffs) Method Number 27.1.2 ; 4-th Auxiliary supply 1997 ; VDLUFA ISBN 3-922712-66-7, in German

[4] Engelen et al. (1994) J. AOAC Int., 77, 760-764

[5] CEN-method draft: Animal feeding stuffs – Determination of phytase activity; Working document N 347 of CEN TC 327

[6] Gisele Gizzi and Christoph von Holst (2005) *Validation study on a new method for the determination of phytase activity in feed: Results from an interlaboratory study conducted according to the IUPAC harmonised protocol*. European Commission, DG JRC, IRMM Geel

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Saxon National Institute for Agriculture, Department of Agricultural Analysis, Leipzig, Germany.